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Characterization of the global structure of low methoxyl pectin in solution☆

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ABSTRACT

Low methoxyl citrus pectin (LMP) and amidated low methoxyl pectin (LMAP) were characterized by high performance size exclusion chromatography (HPSEC) with online light scattering (LS), intrinsic viscosity (η_w), differential refractive index (dRI) and ultra-violet (UV) detection, by amino acid analysis (AAA) and by atomic force microscopy (AFM). HPSEC revealed the following: the weight average molar mass (M_w), ranged 103–288 × 10^3 Da, (η_w), ranged 2.69–4.27 dL/g, radius of gyration (Rg_z), was 28.6–49.5 nm and hydrated radius (Rh_{zv}), was 26.3–41.8 nm. The presence of phenylalanine and tyrosine residues as shown by the UV absorbance at 278 nm in the high molar mass range is indicative of protein or fragments associated with LMP derived from citrus fruit. The ρ value (Rg_z/Rh_{zv}) as a function of molar mass indicated that high molar mass pectin was more compact in shape than intermediate molar mass pectin. AFM images in this study and earlier studies indicate that high methoxyl pectin (HMP) and LMP form network structures in aqueous solution. Unlike HMP networks, LMP networks do not dissociate when dissolved in water at concentrations that are less than 6.6 μg/mL. Furthermore, AFM images, M_w and η_w values for LMP and LMAP indicate that little or no added sugar is bound.

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1. Introduction

Pectin is a complex anionic polysaccharide found in the cell walls of many plants, and is comprised of several different covalently linked polysaccharides (Vincken et al., 2003). It has been found to contain at least 17 different mono-saccharides in various plants. Nonetheless, the major constituent common to all pectin is homogalacturonan (HG), *i.e.* (1–4) linked α-D-galacturonic acid and its methyl ester (Carpita & McCann, 2000). An early study of sodium polygalacturonate in aqueous solution, a fragment of pectin and possibly HG, found that it was highly asymmetric (Fishman, Pepper, & Barford, 1984). The second most abundant constituent in pectin is rhamnogalaturonan I. This constituent contains arabinan, galantan and arabino-galactan side chains. A more detailed description of pectin structure at the mono-saccharide level can be found elsewhere (Kirby, MacDougall, & Morris, 2008).

Electron microscope images obtained by rotary shadowing of peach pectin revealed that pectin isolated from water formed network structures (Fishman, Cooke, Hotchkiss, & Damert, 1993; Fishman et al., 1992). Dissociation of these structures into their component parts in aqueous solution containing 0.05 M NaCl or 50% glycerol revealed that the network subunits were comprised of rods, segmented rods and kinked rods. Electron microscope images confirmed earlier measurements from curve fitting of high performance size exclusion chromatography (HPSEC) chromatograms that pectin structure was comprised of subunits (Fishman, Gillespie, Sondney, & El-Atawy, 1991; Fishman, Gross, Gillespie, & Sondey, 1989). Furthermore, images obtained by Atomic Force Microscopy (AFM) of citrus pectin isolated from water, also revealed that network structures were present at a concentration of 13.1 μg/mL. At a concentration of 6.1 μg/mL the networks were dissociated

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into rods, segmented rods and kinked rods. In addition, dense spherical and "Y" shaped shape structures were observed (Fishman, Cooke, Chau, Coffin, & Hotchkiss, 2007). Similar structures have also been observed in AFM images of tomato (Kirby et al., 2008) and sugar beet pectin (Fishman, Chau, Cooke, & Hotchkiss, 2008).

Commercial pectin, which typically is extracted from apple pomace or citrus peels, has a degree of methyl esterification (DE) of 70–80% (May, 1990; Voragen, Pilnik, Thibault, Axelos, & Renard, 1995) and has been labeled high methoxyl pectin (HMP). HMP has food and pharmaceutical uses some of which include formation of sweet, fruit flavored gels, stabilization of fermented or acidified dairy products, wound bandages and gaskets for receptacles for intestinal fluids from stomatized patients (Rolin, 2002).

Low methoxyl pectin (LMP), DE $\leq$ 50% is obtained by de-esterification of HMP with acid or ammonia in an alcoholic medium (Axelos & Thibault, 1991). Acid deesterification produces LMP with a random distribution of carboxyl groups whereas ammonia produces a blockwise distribution of amide groups and randomly distributed free carboxyl group. Pectin deesterified by salt independent poly methyl esterase from orange produces LMP with a blockwise distribution of carboxyl groups and without significant depolymerization (Hotchkiss et al., 2002; Ström et al., 2007). Both HMP and LMP can undergo gelation but the mechanisms differ. HMP undergoes gelation in the presence of high concentrations of sucrose (e.g. 65%) and low pH (e.g. $\leq$3.5). Gelation has been attributed to a combination hydrogen bond formation and hydrophobic interactions (Li, Al-Assaf, Fang, & Phillips, 2013). In the case of LMP, it has been suggested that the addition of divalent calcium ions holds together two or more polyuronates chains to form side by side aggregates which have been likened to egg boxes (Grant, Morris, Rees, Smith, & Thom, 1973). Presumably, these aggregates are the precursors to gel formation which occurs in the presence of calcium or other divalent cations. It has been found that at low pH (e.g. pH 2.5), that gel formation will occur without the addition of extraneous calcium although the presence of trace amounts of endogenous calcium may play a role (Gilsenan, Richardson, & Morris, 2000).

Low methoxyl and amidated pectin are incorporated in low sugar products, reduced sugar preserves, fruit preparations for yogurts, desert gels and toppings, and savory applications (e.g. sauces and marinades). Furthermore, they are used in low acid, high sugar preserves containing figs, bananas and other confectionary products (Anonymous, 2009).

The objective of this study is to determine the effect of added sucrose and to some extent endogenous proteins on the structure of LMP by employing HPSEC with multi online detectors and by atomic force microscopy (AFM). Furthermore, measurement of molar mass, viscosity, radii and AFM images of LMP in water will provide additional structural information on the tendency of pectin to aggregate in the presence and absence of sugar, and without added calcium or other divalent cations. The ability of pectin to aggregate is vital in the ability of LMP to form gels. Therefore, the knowledge gained in this study should be useful in the formulation of LMP gels.

2. Materials and methods

2.1. Materials

Six low methoxyl pectin samples were gifts from CP Kelco, a Huber Company (www.cpkelco.com). They were labeled LMP 36.1, LMP 36.8, LMP 50.2, LMAP 1, LMAP 2 and LMAP 3. Two low methoxyl pectin samples were gifts from DuPont/Danisco (www.danisco.com), and were labeled LMP 32 and 48. All samples were standardized with sugar by the supplier. Therefore, LMP 36.8 was also characterized with sugar removed by both methanol and isopropanol. Samples labeled as LMP samples were not amidated whereas LMAP samples were amidated. The number following LMP samples is the degree of methyl esterification (DE) whereas in the case of LMAP samples, the end number merely indicates different samples. LMP 36.8 was labeled LMP IPA ppt 36.8 or LMP MeOH ppt 36.8 depending on whether sugar was removed from pectin by precipitating it with isopropyl alcohol (IPA) or methanol (MeOH). Either 2.5 g of pectin was added to a 100 mL of 70/30 MeOH/H_2O solution or 5.5 g of pectin was added to 100 g 70/30 IPA solution, stirred overnight and passed through a Whatman paper filter (Grade 541, Sigma–Aldrich, St. Louis, MO). The precipitated pectin was removed from the filter and dried overnight at room temperature in a vacuum oven.

2.2. High performance size exclusion chromatography

Dried samples of low methoxy pectin (2 mg/mL) were dissolved in mobile phase (0.05 M $NaNO_3$ and 0.01% NaN_3) and filtered through a 0.45 μm Millex HV filter (Merck Ltd, Tullagreen, Ireland). The solvent delivery system consisted of a model 1200 series degasser, auto sampler and a pump (Agilent Technologies, Germany). The flow rate was 0.7 mL/min. The injection volume was 200 μL. Samples were run in triplicate. The column set consisted of three model TSK $GMPW_{xl}$, 7.8 × 300 mm, 13 μm particle size exclusion columns (Tosoh Bioscience, Tokyo, Japan) in series. Placed directly before and after the column set were TSK_{gel} PW_{xL} guard columns (Tosoh Bioscience, Tokyo, Japan), dimensions 6.0 mm I.D. × 4.0 cm, pore size 12 μm. The columns were in a heated water bath set at 35 °C. The column set was connected online and in series to a UV-1260 Infinity spectrophotometer (Agilent Technologies, Germany), HELEOS II multi-angle laser light scattering photometer (MALLS) (Wyatt Technology, Santa Barbara, CA), model 255-V2 differential pressure viscometer (DP) and a differential refractive index detector (RI) (Wyatt Technology, Santa Barbara, CA). The UV/VIS, MALLS, DP and dRI detectors were aligned with Bovine Serum Albumin (BSA) and normalized with Pullulan 50. The extinction coefficient for the UV wavelength at 278 nm was determined from the RI concentration of each individual sample. The d*n*/d*c* value used was 0.132. Electronic outputs from all the scattering angles measured by the UV, MALLS, DP and dRI were sent to a desktop computer directory for processing with ASTRA (Ver. 6.1.1.17) software (Wyatt Technology, Santa Barbara, CA).

2.3. Amino acid analysis

Dehydrated pectin samples were hydrolyzed in 6 N HCl containing a small amount of phenol. The hydrolysis flasks were extensively purged of oxygen using a PicoTag workstation (Waters Corp., Milford, MA), and then incubated at 110 °C for 20 h. Hydrolyzed samples were filtered, dried under vacuum, and derivatized with AccQFluor reagent (Waters) following the manufacturer's directions. Chromatography was performed using procedures described as 'system 1' in Cohen and De Antonis (Cohen & De Antonis, 1994), with α-aminobutyric acid as an internal standard. Separation was achieved using an AccQTag C18 reverse phase column (Waters); detection by fluorescence used 250 nm as the excitation and 395 nm as the emission wavelength. Hydrolysis, derivativzation and analysis of each sample were performed in triplicate. The total percentage of nitrogen and crude protein present in low methoxyl pectin as determined by Kjeldahl analysis has been described previously (Fishman, Chau, Cooke, Yadav, & Hotchkiss, 2009).

Table 1
Selected properties of low methoxyl pectin samples.

Samples	%Protein[a]	%N[b]	%AAPN[c]	pH[d]	%Sugar[e]
LMP 32	1.55 (0.02)	1.46 (0.01)	0.25	4.01	28.4
LMP 36.1	0.89 (0.01)	0.13 (0.01)	0.14	3.43	38.9
LMP 36.8	1.17 (0.01)	0.15 (0.01)	0.18	3.47	36.6
LMP 36.8 IPA ppt		0.23 (0.05)		3.36	0.6
LMP 36.8 MeOH ppt		0.22 (0.01)		3.35	2.2
LMP 48	1.56 (0.01)	1.18 (0.06)	0.24	4.22	39.8
LMP 50.2	1.54 (0.02)	0.38 (0.01)	0.25	5.66	26.0
LMAP 1	1.66 (0.05)	1.63 (0.07)	0.27	5.40	42.6
LMAP 2	1.35 (0.07)	1.63 (0.1)	0.22	5.83	44.2
LMAP 3	1.45 (0.04)	1.81 (0.08)	0.23	5.56	42.2

[a] %Protein based on amino acid content.
[b] %Total nitrogen from Kjeldahl analysis.
[c] %Protein nitrogen obtained by dividing protein based on amino acid content by 6.25.
[d] pH values were determined for pectins dissolved in water containing 0.05 M $NaNO_3$ and 0.01% NaN_3 at a concentration of 2 mg/mL.
[e] Calculated by subtracting the weight of recovered pectin from the total weight of solid sample injected into the column set as determined by HPSEC.

Table 3
Molar masses (M_w) and intrinsic viscosities (η_w) of low methoxyl pectin samples.

Samples	%Rec[a]	$M_w \times 10^{-3}$	M_w/M_n[b]	η_w (dL/g)
LMP 32	71.6 (0.2)	107 (2)	1.81 (0.03)	2.69 (0.01)
LMP 36.1	61.1 (2.0)	103 (4)	2.17 (0.08)	2.86 (0.08)
LMP 36.8	63.4 (1.0)	118 (2)	2.18 (0.04)	3.10 (0.03)
LMP 36.8 IPA ppt	99.4 (1.0)	110 (2)	2.08 (0.01)	3.05 (0.01)
LMP 36.8 MeOH ppt	97.8 (1.0)	122 (6)	2.18 (0.04)	3.01 (0.01)
LMP 48	60.2 (0.6)	152 (1)	1.86 (0.02)	4.27 (0.03)
LMP 50.2	74.0 (0.1)	124 (1)	2.16 (0.01)	3.33 (0.01)
LMAP 1	57.4 (0.1)	288 (5)	2.40 (0.06)	4.17 (0.01)
LMAP 2	55.8 (1.0)	221 (10)	2.35 (0.09)	3.98 (0.10)
LMAP 3	57.8 (0.7)	236 (6)	2.14 (0.04)	3.91 (0.05)

[a] Percent pectin recovered.
[b] Polydispersity of molecular weight.

2.4. Atomic force microscopy

Samples of dry pectin powder were weighed, dissolved in distilled water and diluted serially from a 10 mg/mL stock solution to 10 μg/mL. Two μL aliquots of the pectin solutions were spotted onto freshly cleaved mica disks and air dried for ½ hour. Atomic force microscopy was done on micrometer-size areas of the dried samples using TESP and TESPA cantilevers in the soft tapping mode in air with a Dimension FastScan NanoScope 5 Microscope platform (Bruker Nano Surfaces Division, Santa Barbara, CA).

3. Results and discussion

Table 1 includes some important properties for LMP and LMAP. Sucrose is often added to produce standardized jelling properties that tend to change with variations in climatic growing conditions of the source plant. The percentage of protein and protein nitrogen compared to the percentage of total nitrogen allows for a more accurate knowledge of nitrogen in amidated pectin. Since the flavor of a food is related to its pH, it is of interest to know how additives such as LMP or LMAP might affect the pH of the food. Table 1 contains pH values for pectins dissolved in water containing 0.05 M $NaNO_3$ and 0.01% NaN_3 at concentration of 2 mg/mL. These pectin solutions were identical to those that were injected into the HPSEC columns. As shown in Table 1, protein content calculated from amino acid content ranged from 0.89 to 1.66%, nitrogen content ranged from 0.13 to 1.81%, protein nitrogen obtained by dividing protein content by 6.25 ranged from 0.14 to 0.27, pH ranged from 3.36 to 5.83 and sucrose content ranged from 0 to 42 %. It appears that most if not all the nitrogen in LMP 36.1, LMP 36.8 and LMP 50.2 came from protein whereas LMP 32 and LMP 48 had an appreciable amount of non-protein nitrogen. We conclude this because LMP 36.1, LMP 36.8 and LMP 50.2 had values of nitrogen calculated from amino acid analysis and Kjeldahl analysis which were fairly close. Whereas in the case of LMP 32 and LMP 48 the values of nitrogen calculated from amino acid analysis were much smaller than those obtained from Kjeldahl analysis. The amidated pectin had higher percentages of nitrogen than LMP 36.1, LMP 36.8 and LMP 50.2. In the cases of LMP 32 and LMP 48, for unknown reasons, the total N was higher than expected for a non-amidated low methoxyl pectin. The amidated pectin was higher in pH than non-amidated pectin except for LMP 50.2.

Table 2 contains the amino acid analysis of the various pectin samples. With the exception of a few amino acids in a few of the samples, the amino acid content and most probably the protein content in all the samples are comparable. The exception is that the amidated pectin samples had lower ASN and VAL values but higher PRO values than the non-amidated samples. The content of phenylalanine and tyrosine residues is sufficient to allow

Table 2
Amino acid composition in terms of number/1000 amino acids (±1 standard deviation) and protein content of commercial low methoxyl pectin products.

Amino acid	LMP 32	LMP 36.1	LMP 36.8	LMP 48	LMP 50.2	LMAP 1	LMAP 2	LMAP 3
ALA	72.9 (0.6)	82.4 (0.5)	78.2 (0.7)	76.2 (0.7)	80.3 (1.5)	74.4 (1.1)	80.2 (1.2)	78.0 (1.2)
ARG	50.1 (0.1)	42.0 (0.4)	51.1 (0.5)	44.2 (0.5)	44.4 (0.2)	40.8 (0.5)	40.2 (0.4)	48.0 (0.5)
ASX[a]	101.1 (0.9)	111.4 (0.7)	106.6 (0.6)	108.3 (1.0)	106.3 (0.3)	89.8 (0.2)	93.8 (0.0)	84.5 (0.6)
CYS	3.1 (0.4)	0.8 (0.2)	1.0 (0.5)	1.5 (1.3)	1.9 (0.9)	0.4 (0.4)	5.7 (7.6)	1.1 (1.3)
GLX[b]	124.4 (0.2)	110.5 (0.4)	139.1 (0.2)	113.5 (0.4)	121.4 (0.7)	116.3 (0.8)	117.1 (2.5)	117.2 (0.3)
GLY	105.4 (0.1)	101.6 (2.0)	99.1 (0.6)	99.7 (0.6)	97.8 (0.4)	90.1 (0.4)	95.9 (1.0)	101.0 (1.9)
HIS	37.0 (0.2)	28.4 (0.1)	27.2 (0.1)	33.3 (0.2)	27.7 (0.2)	29.5 (0.6)	30.6 (0.5)	30.9 (0.7)
ILE	41.6 (0.1)	51.0 (0.2)	51.2 (0.1)	45.9 (0.5)	50.2 (0.1)	44.5 (0.3)	44.5 (0.2)	43.0 (0.6)
LEU	69.5 (0.1)	83.1 (0.2)	85.6 (0.5)	74.2 (0.5)	82.9 (0.3)	74.4 (0.4)	76.0 (1.0)	72.1 (0.8)
LYS	75.1 (0.6)	64.2 (0.9)	59.1 (0.7)	74.1 (0.9)	58.0 (0.7)	69.9 (1.2)	76.2 (1.2)	75.4 (1.4)
MET	3.1 (0.4)	2.4 (0.3)	2.9 (0.6)	2.8 (0.4)	5.0 (2.9)	3.4 (0.9)	4.5 (0.2)	3.1 (2.7)
PHE	40.3 (0.2)	48.0 (0.4)	46.0 (0.3)	44.2 (0.8)	46.7 (0.4)	46.8 (0.1)	43.4 (0.7)	40.9 (0.8)
PRO	66.8 (0.0)	68.2 (0.0)	63.8 (0.2)	66.7 (0.4)	64.5 (1.7)	116.0 (1.8)	77.7 (1.2)	91.7 (8.3)
SER	58.6 (0.0)	60.8 (0.7)	59.1 (0.2)	60.2 (0.9)	60.6 (0.2)	58.6 (0.4)	63.2 (0.5)	61.1 (1.1)
THR	46.8 (0.3)	53.3 (0.2)	49.0 (0.4)	49.0 (0.5)	50.3 (2.6)	46.7 (0.4)	51.7 (0.2)	50.0 (1.1)
TYR	35.4 (0.4)	16.5 (0.3)	6.1 (0.1)	34.9 (1.3)	30.2 (0.7)	33.7 (0.1)	35.0 (1.3)	37.7 (1.9)
VAL	68.9 (0.0)	75.3 (0.2)	74.9 (0.2)	71.4 (0.2)	71.9 (0.0)	64.6 (0.3)	64.3 (0.8)	64.5 (2.9)
%Protein (dry basis)	1.55 (0.02)	0.89 (0.01)	1.17 (0.01)	1.56 (0.01)	1.54 (0.12)	1.66 (0.05)	1.35 (0.07)	1.45 (0.04)

[a] ASX includes ASP + ASN.
[b] GLX includes GLU + GLN.

Table 4
RMS radius of gyration (Rg_z) and hydrated radius (Rh_{zv}) of low methoxyl pectin.

Samples	Rg_z	Rh_{zv}	$\rho = Rg_z/Rh_{zv}$
LMP 32	28.6 (2.0)	26.3 (0.1)	1.09
LMP 36.1	32.2 (2.0)	27.1 (1.0)	1.19
LMP 36.8	32.3 (0.3)	28.4 (0.4)	1.14
LMP 36.8 IPA ppt	33.6 (0.5)	26.3 (0.6)	1.28
LMP 36.8 MeOH ppt	40.8 (3.0)	28.9 (1.0)	1.42
LMP 48	37.3 (0.4)	31.0 (0.5)	1.20
LMP 50.2	34.6 (0.8)	29.3 (0.1)	1.18
LMAP 1	49.5 (1.0)	41.8 (0.2)	1.18
LMAP 2	41.9 (2.0)	37.2 (0.2)	1.13
LMAP 3	42.3 (0.7)	37.4 (0.3)	1.13

measurement of their UV absorbance at 278 nm. Thus this wavelength was employed to obtain the HPSEC chromatogram of the pectin/protein complex.

Table 3 contains weight average molar masses (M_w), polydispersities (M_w/M_n) and weight average intrinsic viscosities (η_w) of pectin with and without amidation. M_w and root mean square radius of gyration (Rg_z) which is located in Table 4, were obtained simultaneously from the MALLS by measuring the excess scattering intensity due to a diluted solution of dissolved macromolecules as a function of scattering angle (Fishman, Walker, Chau, & Hotchkiss, 2003; Wyatt, 1993). In this study, amidated pectins have higher molar masses and polydispersities than non-amidated pectins. With the exception of LMP 48, amidated pectins have larger intrinsic viscosities than non-amidated pectin, which is consistent with their higher molar masses.

Representative chromatograms in Fig. 1A–C are for samples LMP 36.8, LMP 36.8 IPA ppt., and LMAP 1, respectively. These chromatograms are for pectin with sugar, without sugar and amidated pectin with sugar, respectively. In all samples, high molar mass material elutes between about 17 and 26 mL as indicated by the presence of appreciable light scattering (LS) and viscosity (DP)

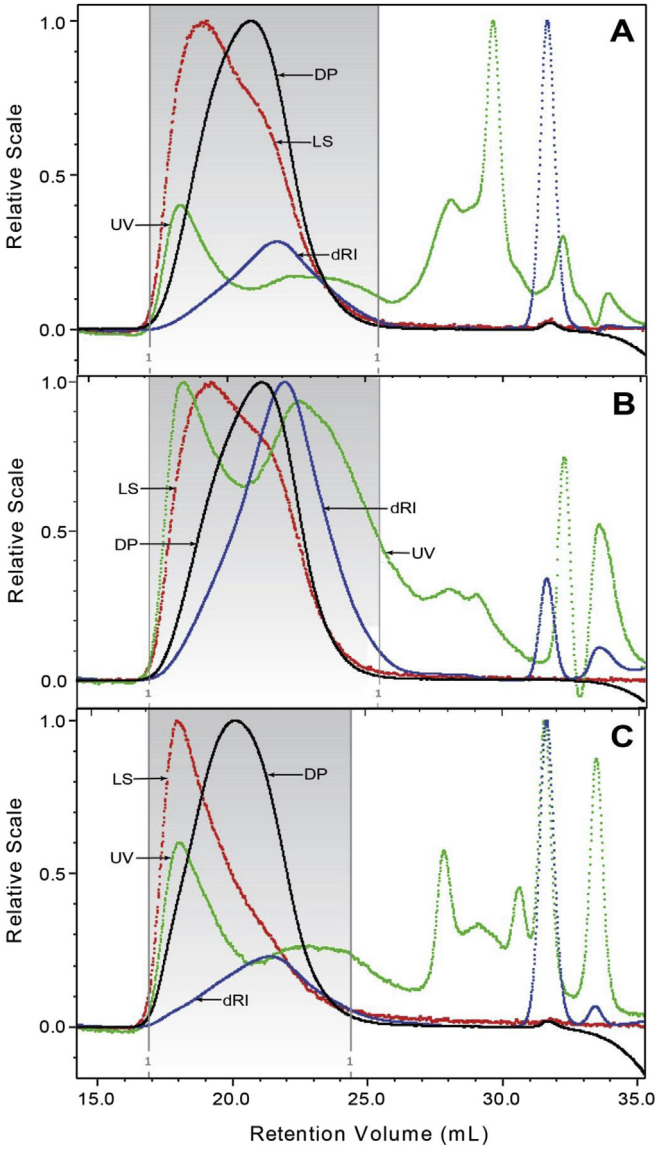

Fig. 1. HPSEC chromatograms of low methoxyl pectin: (A) LMP 36.8, (B) LMP 36.8 IPA ppt, (C) LMAP 1. Superimposed chromatograms were obtained from: 90° light scattering (LS); viscosity (DP); UV 278 nm (UV) and refractive index (dRI) detectors.

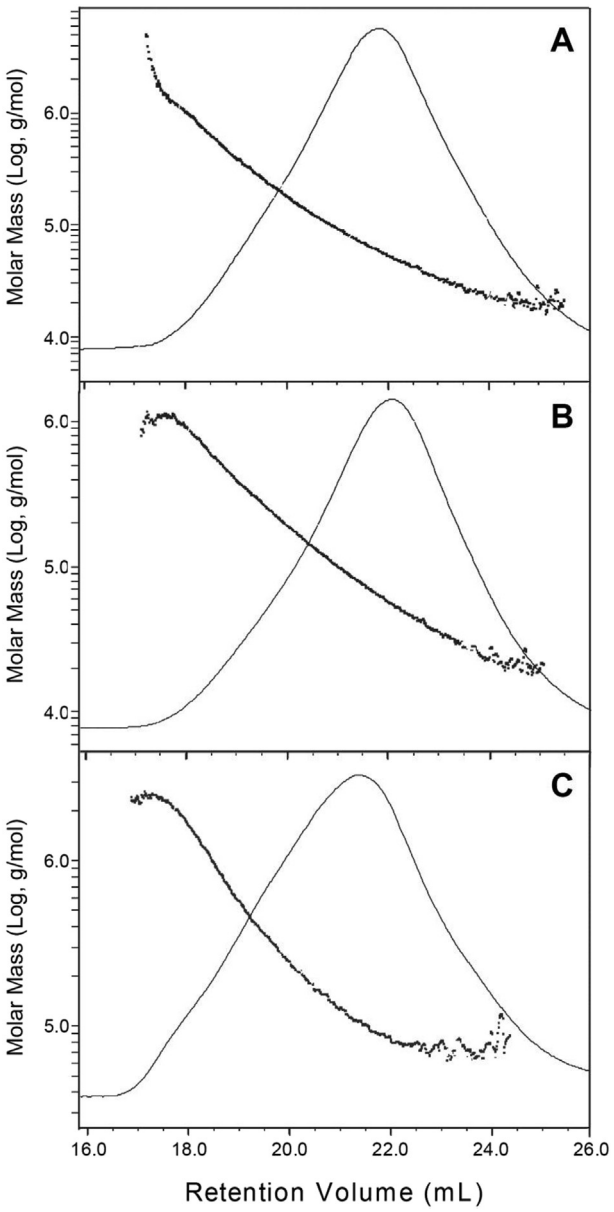

Fig. 2. Molar mass calibration curves superimposed on HPSEC chromatograms of low methoxyl pectin: (A) LMP 36.8; (B) LMP 36.8 IPA ppt; and (C) LMAP 1. Calibration curves and chromatograms were obtained from the refractive index (dRI) detector.

traces, whereas the low molar mass material elutes between about 26 and 35 mL as indicated by the absence of light scattering and a barely visible viscosity peak which is only present when sugar is present. Furthermore, all the components in the light scattering (LS), intrinsic viscosity (η_w), and refractive index (dRI) traces overlap into a single, broad, multi-component peak whereas all the components in the ultra-violet (UV) trace are encompassed within two broad, multi-component peaks. The relative concentration of the proteinacous material associated with the first broad peak is higher than the second one in all three figures. This indicates that a higher concentration of proteinacous material is associated with the higher molar mass pectin than with lower molar mass pectin. Nevertheless, based on relative areas of the two peaks, a larger fraction of proteinacous material is associated with the lower molar mass pectin than with higher molar mass pectin. Also there is an appreciable fraction of UV absorbing material visible in the low molar mass range where there is no light scattering visible. On the other hand, there is a negligible dRI signal in the reliable portion of low molar mass range. Possibly, the UV signal is due to a trace amount of absorbing amino acids. The dRI signal arising between 31 and 33 mL in Figs. 1 and 3 is probably due to a mixture of sucrose and monosaccharide fragments from pectin.

Fig. 2A–C contains dRI calibration curves in which molar mass is plotted against elution time and is superimposed on the dRI chromatograms for the LMP 36.8, LMP 36.8 IPA ppt, and LMAP 1 pectin samples respectively. All the pectin characterized in this study showed similar behavior with respect to the behavior of the calibration curves found in Fig. 2.

Table 4 contains the rms radius of gyration (Rg_z) and the hydrated radius obtained from viscosity (Rh_{zv}). Rh_{zv} was calculated according to the Einstein–Simha relation, Equation (1) (Armstrong, Wenby, Meiselman, & Fisher, 2004).

$$Rh_z = (3M\eta_z/10\pi N_A)^{1/3} \quad (1)$$

Where: η_z is the intrinsic viscosity and N_A is Avagadro's number.

Also, Table 4 contains the ratio, Rg_z/Rh_z (ρ). The structural significance of the parameter, ρ, has been explored by Burchard (1996). The ρ parameter allows one to compare the relative extension of two macromolecules dissolved under identical conditions. The larger the value of ρ is, the more extended the

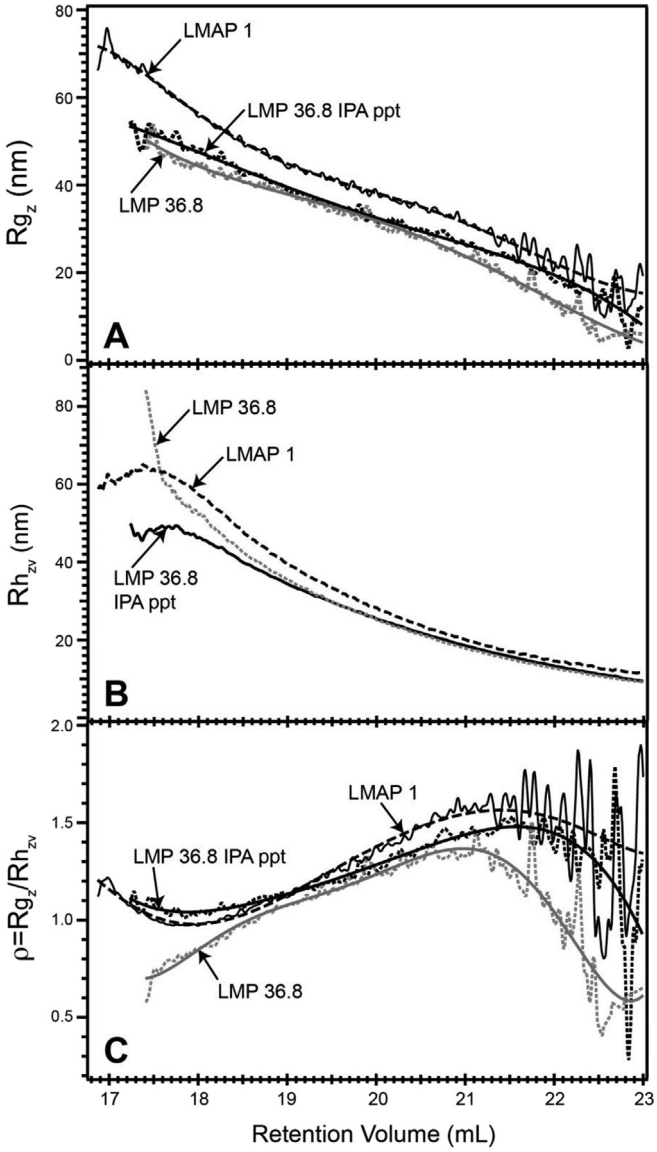

Fig. 3. Change in radii as a function of retention volume for low methoxyl pectin. (A) rms radius of gyration (Rg_z), (B) hydrated radius (Rh_{zv}) and (C) ρ (Rg_z/Rh_{zv}).

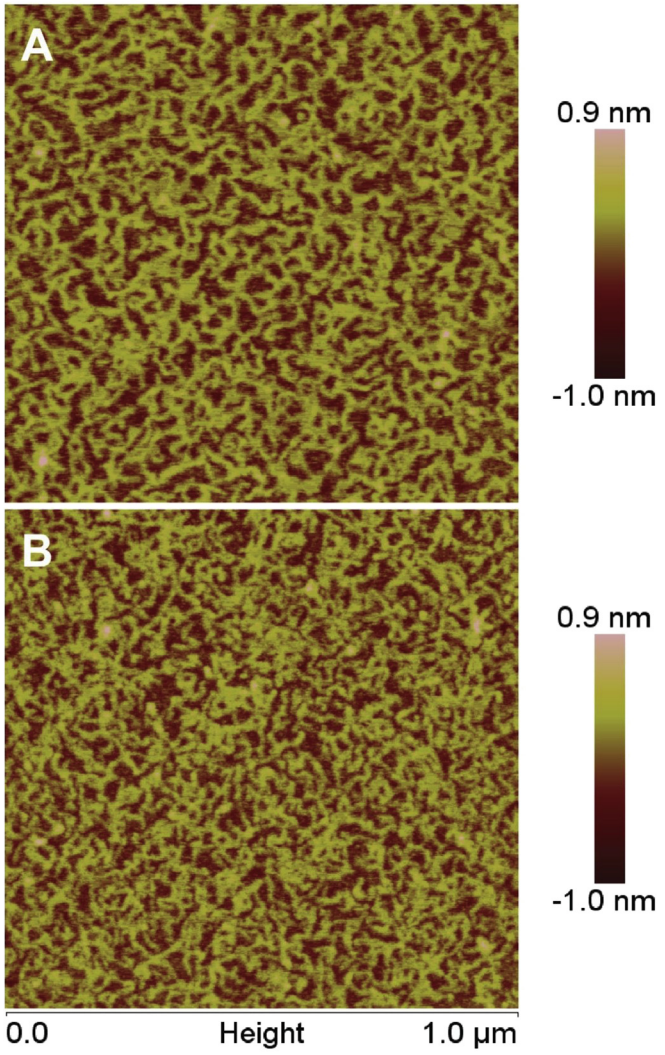

Fig. 4. AFM height image of low methoxyl pectin dissolved in water, deposited on mica and air dried: (A) LMP 36.8 contained 7.4 μg/mL of pectin and 3.6 μg/mL of sucrose, (B) LMP 36.8 IPA ppt contained 10 μg/mL of pectin.

macromolecule is. In this study, Measurement of concentration in the macromolecular range as shown by chromatograms in Fig. 1 reveals that macromolecules measured by UV elute before those measured by dRI. RI measurements of concentration may include macromolecules both associated and not associated with protein whereas UV measurements only include pectin associated with protein. Thus, one may conclude from these results that pectin associated with protein is more extended than pectin not associated with protein.

Fig. 3A–C shows the change in Rg_z, Rh_{zv} and ρ (Rg_z/Rh_{zv}) as retention volume increases or molar mass decreases as shown in Fig. 2A–C. As shown by Fig. 3A, Rg_z, decreases with decrease in molar mass. For the most part, as found in Fig. 3B, Rh_{zv} also decreases with decreases in molar mass. On the other hand, for the most part, ρ appears to pass through maxima with decreases in molar mass. Thus, at retention volumes from about 18.5 mL to about 21 mL, the shape of the three pectins tend to become more extended with decreasing molar mass. Whereas beyond 21 mL, the shape of the three pectins become less extended with decreasing molar mass.

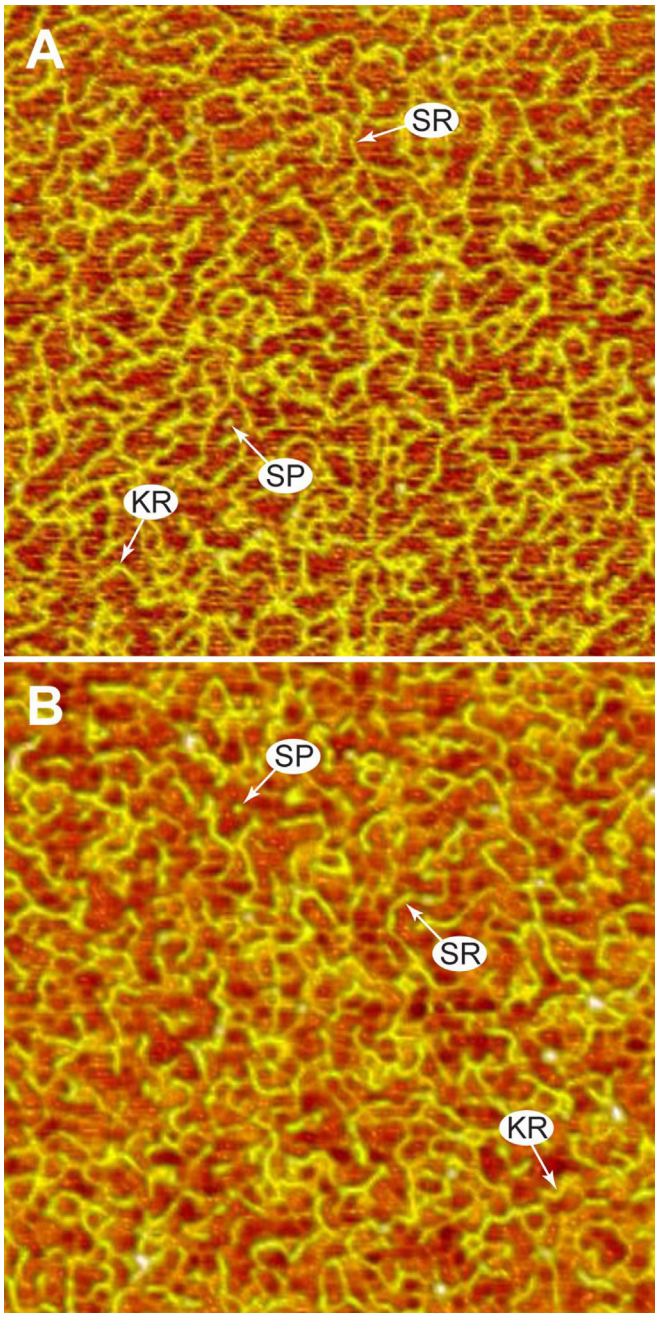

Fig. 5. 3D AFM images of pectin networks deposited on mica from aqueous solutions containing pectin (A) LMP 36.8 contained 3.2 μg/mL of pectin and 1.8 μg/mL of sucrose. (B) LMP 36.8 IPA ppt contained 5 μg/mL of pectin. Labels: Rod (SR); (SP) Sphere; (KR) Kinked Rod.

Fig. 4A and B is height images obtained by Atomic Force Microscopy (AFM). Fig. 4A contained 7.4 μg/mL of pectin and 3.6 μg/mL sucrose whereas Fig. 4B contained 10 μg/mL of pectin alone. Pectin in each figure was found to form a network structure. Even though Fig. 4A contained about one third less pectin than Fig. 4B, the spaces within networks did not appear appreciably different. Previously AFM images of high methoxyl pectin (HMP) in the presence and absence of sucrose (Fishman et al., 2007; Fishman, Cooke, & Coffin, 2004) were found to form network structures. In the case of HMP, lowering its concentration to 6.6 μg/mL dissociated the network into its component parts. Fig. 5A and B contains 3D images of pectin networks deposited on mica from aqueous solutions containing pectin at concentrations of 5 and 3.2 μg/mL respectively. Fig. 5B also contains 1.8 μg/mL of sucrose. Apparently interactions holding LMP networks together are stronger than those holding HMP networks together. This difference in strength can be attributed to LMP having more groups capable of hydrogen bonding than HMP. As in the case of HMP, the individual strands comprising the networks of LMP (see Fig. 5) are combinations of segmented rods (SR), kinked rods (KR), Spheres (SP) and possibly rods.

4. Conclusions

Protein or fragments thereof associated with low methoxyl pectin derived from citrus fruit appear to co-elute with pectin moieties of all sizes. Co-elution will occur if protein or fragments thereof interact due to covalent, hydrogen or ionic bonding. Co-elution also will occur when there is no interaction but if co-elution is merely due to size equality. Based on this and previous AFM images it appears that both high and low methoxyl pectin are capable of forming network structure in aqueous solution. Nonetheless, LMP networks tend to resist dissociation at lower concentration in solution more than HMP networks. Furthermore, the thickness of pectin molecule moieties in the presence and absence of sugar appear not to be different at the nanometer level for pectin with a 36.8% degree of esterification. This may indicate little or no sugar is bound to the pectin. Moreover, the pectin with 36.8% degree of esterification exhibited intrinsic viscosities and molar masses which were unchanged regardless of the presence or absence of sugar.

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References

Anonymous. (2009). *Non amidated low methoxyl pectin*. Technical evaluation report (pp. 1–11). USDA. CAS number: 9000-69-5, INS no. 440.

Armstrong, J. K., Wenby, H. J., Meiselman, H. J., & Fisher, T. C. (2004). The hydrodynamic radii of macromolecules and their effect on red blood cell aggregation. *Biophysical Journal, 87*(6), 4259–4270.

Axelos, M. A. V., & Thibault, J. F. (1991). The chemistry of low-methoxyl pectin gelation. Ch. 6. In R. H. Walter (Ed.), *The chemistry and technology of pectin* (pp. 109–118). San Diego, CA: Academic Press, Inc.

Burchard, W. (1996). Combined static and dynamic light scattering. Ch. 13. In W. Brown (Ed.), *Light scattering: Principles and development* (pp. 439–475). Oxford, UK: Clarendon Press.

Carpita, N., & McCann, M. C. (2000). Biochemistry and molecular biology of plants. In B. B. Buchanan, W. Gruissem, & R. L. Jones (Eds.), *Biochemistry and molecular biology of plants* (pp. 52–108). Rockville, MD: American Society of Plant Physiologists.

Cohen, S. A., & De Antonis, K. M. (1994). Applications of amino acid derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate: analysis of feed grains, intravenous solutions and glycoproteins. *Journal of Chromatography A, 661*(1–2), 25–34.

Fishman, M. L., Chau, H. K., Cooke, P. H., & Hotchkiss, A. T., Jr. (2008). Global structure of microwave-assisted flash-extracted sugar beet pectin. *Journal of Agricultural and Food Chemistry, 56*(4), 1471–1478.

Fishman, M. L., Chau, H. K., Cooke, P. H., Yadav, M. P., & Hotchkiss, A. T., Jr. (2009). Physico-chemical characterization of alkaline soluble polysaccharides from sugar beet pulp. *Food Hydrocolloids, 23*(6), 1554–1562.

Fishman, M. L., Cooke, P. H., Chau, H. K., Coffin, D. R., & Hotchkiss, A. T., Jr. (2007). Global structures of high methoxyl pectin from solution and in gels. *Biomacromolecules, 8*(2), 573–578.

Fishman, M. L., Cooke, P. H., & Coffin, D. R. (2004). Nanostructure of native pectin sugar acid gels visualized by atomic force microscopy. *Biomacromolecules, 5*(2), 334–341.

Fishman, M. L., Cooke, P. H., Hotchkiss, A. T., Jr., & Damert, W. (1993). Progressive dissociation of pectin. *Carbohydrate Research, 248*, 303–316.

Fishman, M. L., Cooke, P. H., Levaj, B., Gillespie, D. T., Sondey, S. M., & Scorza, R. (1992). Pectin microgels and their subunit structure. *Archives of Biochemistry and Biophysics, 294*(1), 253–260.

Fishman, M. L., Gillespie, D. T., Sondney, S. M., & El-Atawy, Y. S. (1991). Intrinsic viscosity and molecular weight of pectin components. *Carbohydrate Research, 215*(1), 91–104.

Fishman, M. L., Gross, K. C., Gillespie, D. T., & Sondey, S. M. (1989). Macromolecular components of tomato fruit pectin. *Archives of Biochemistry and Biophysics, 274*(1), 179–191.

Fishman, M. L., Pepper, L., & Barford, R. A. (1984). Degree of polymerization of sodium polygalacturonate by membrane osmometry. *Journal of Polymer Science: Polymer Physics Edition, 22*(5), 899–901.

Fishman, M. L., Walker, P. N., Chau, H. K., & Hotchkiss, A. T. (2003). Flash extraction of pectin from orange albedo by steam injection. *Biomacromolecules, 4*(4), 880–889.

Gilsenan, P. M., Richardson, R. K., & Morris, E. R. (2000). Thermally reversible acid-induced gelation of low-methoxy pectin. *Carbohydrate Polymers, 41*(4), 339–349.

Grant, G. T., Morris, E. R., Rees, D. A., Smith, P. J. C., & Thom, D. (1973). Biological interactions between polysaccharides and divalent cations: the egg-box model. *FEBS Letters, 32*(1), 195–198.

Hotchkiss, A. T., Jr., Savary, B. J., Cameron, R. G., Chau, H. K., Brouillette, J., Luzio, G. A., et al. (2002). Enzymatic modification of pectin to increase its calcium sensitivity while preserving its molecular weight. *Journal of Agricultural and Food Chemistry, 50*(10), 2931–2937.

Kirby, A. R., MacDougall, A. J., & Morris, V. J. (2008). Atomic force microscopy of tomato and sugar beet pectin molecules. *Carbohydrate Polymers, 71*(4), 640–647.

Li, X. Y., Al-Assaf, S., Fang, Y., & Phillips, G. O. (2013). Characterisation of commercial LM-pectin in aqueous solution. *Carbohydrate Polymers, 92*(2), 1133–1142.

May, C. D. (1990). Industrial pectins: sources, production and applications. *Carbohydrate Polymers, 12*(1), 79–99.

Rolin, C. (2002). Commercial pectin preparations. In G. B. Seymour, & J. P. Knox (Eds.), *Pectins and their manipulations* (pp. 222–241). USA/Canada: CRC Press, Blackwell Publishing.

Ström, A., Ribelles, P., Lundin, L., Norton, I. T., Morris, E. R., & Williams, M. A. K. (2007). Influence of pectin fine structure on the mechanical properties of calcium-pectin and acid-pectin gels. *Biomacromolecules, 8*(9), 2668–2674.

Vincken, J.-P., Schols, H. A., Oomen, R. J. F. J., McCann, M. C., Ulvskov, P., Voragen, A. G. J., et al. (2003). If homogalacturonan were a side chain of rhamnogalacturonan I. Implications for cell wall architecture. *Plant Physiology, 132*(4), 1781–1789.

Voragen, A. G. J., Pilnik, W., Thibault, J. F., Axelos, M. A. V., & Renard, C. M. G. C. (1995). Pectins. In A. M. Stephen (Ed.), *Food polysaccharides and their applications* (pp. 287–339). New York, NY: Marcel Dekker.

Wyatt, P. J. (1993). Light scattering and the absolute characterization of macromolecules. *Analytica Chimica Acta, 272*, 1–40.